Effect of Heat on Peanut Proteins. I. Solubility Properties and Immunochemical–Electrophoretic Modifications

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The effect *in situ* of wet and dry heat on peanut proteins was investigated by acrylamide electrophoresis and by immunochemical techniques. Only two proteins (from approximately 14 antigenic constituents) remained antigenic after seeds were imbibed in water (40% moisture) and heated at 110°C. Dry heat (<5% moisture), on the other hand, induced sequential antigenic destruction between 110 and 155°C. The major peanut globulin, α-arachin, and one other protein remained antigenic in both wet- and dry-heated seeds within this temperature range. Disc electrophoresis showed greater differences in protein migration for the dry than for the wet-heated seed. A protein solubility *vs.* temperature plot showed that protein solubility was inversely proportional to temperature with dry heat; a sigmoid-like curve was observed after wet heat. The maximum solubility of α-arachin occurred after wet heat at 120°C.

Globular proteins generally consist of several helical and nonhelical (random coil or pleated sheet) regions which vary in different proteins. Thus, heat might be expected to induce unique thermal effects on certain proteins at varying rates and temperatures. Heat has been proposed to dehydrate protein molecules leading to peptide linkages between free amino and carboxyl groups (Haurowitz, 1965). Conversely, cleavage of peptide bonds by heat obtains rearrangements of the peptide chains which can then react with each other or with macromolecules such as lipids and carbohydrates. It has been reported that heat leaves a protein molecule in the zwitterionic state (Haurowitz, 1965), suggesting that both intramolecular hydrogen bonds and apolar bonds are cleaved. The resultant changes in conformation usually make the protein less soluble, modify electrostatic charge, and allow for the formation of complex products. The insolubility of heat-treated proteins has also been ascribed to cleavage of native disulfide bonds (Gorbacheva, 1957).

Over 75% of the peanut proteins are considered globulins. Classic fractionation of the peanut proteins was first reported by Johns and Jones (1916) and by Jones and Horn (1930). Subsequent investigators have described the sedimentation properties (Johnson and Naismith, 1953; Johnson and Shooter, 1950; Johnson *et al.*, 1950), chromatographic and electrophoretic properties (Dechary *et al.*, 1961; Neucere, 1969; Tombs and Lowe, 1967), and immunochemical properties (Daussant *et al.*, 1969a,b) of the major peanut globulins.

The aim of the present study was to investigate by electrophoretic and immunochemical techniques the structural modifications and solubility properties of the proteins extracted from heated whole seeds at two moisture levels.

EXPERIMENTAL

Heat Treatment. Eleven kilograms of dehulled intact Virginia 56-R peanuts, including the testae (1968 crop), were divided into the 1-kg lots. One lot was unheated and served as the control. Five samples were allowed to imbibe distilled water for 16 hr at 25°C to a final moisture content of 40%, placed in ventilated trays, and were heated for 1 hr in a forced draft oven at 110, 120, 130, 145, and 155°C, respectively. Five other samples were heated on an "as is" basis, 5% moisture, under identical conditions.

After equilibration to room temperature, each sample was homogenized in 1:1 hexane–acetone, using 3:1 solvent per kg seeds in a Sorvall Omnimixer for 5 min at 5°C. The homogenate was filtered under vacuum with each batch, yielding approximately 600 g of a fine meal.

Protein Extraction. A portion of each peanut meal (500 mg) (1, control; 2–6, wet heat; 7–11 dry heat) was mixed with 5 ml of 0.072 *M* phosphate buffer, pH 7.9, ionic strength 0.2, and agitated for 2 hr at 25°C. The mixture was clarified by centrifugation at 37,000 × *g* for 30 min. The clear supernatants were isolated from the residue and lipid layer with a syringe and needle. The extractable protein from each meal and relative protein solubilities were determined.

Analytical Methods. Protein content was determined by the method of Lowry *et al.* (1951). Immunoelectrophoresis was performed according to Grabar and Williams (1953) using 1.5% Ionagar gel in 0.25 *M* veronal buffer, pH 8.2, employing the LKB immunoelectrophoretic kit. A voltage gradient of 4 V/cm was applied for 2 hr at room temperature. Each well was filled with 1.5 mg of protein prior to electrophoresis. After separation, each trough was filled twice with antiserum against the total proteins of the peanut.

Disc electrophoresis was carried out according to Davis (1964) employing 7.5% Cyanogum 41 gelling agent (Fisher Scientific Co.) in the running gel (Tris-Glycine buffer, pH 8.4.). The large-pore gel or stacking gel was prepared according to Mikola (1965) (Tris-Borate buffer pH 8.1, 3.3% acrylamide). The Tris-Glycine buffer was diluted 1:10 and used as the running buffer. Electrophoresis was performed at a constant current of 5 mA/tube for approximately 1 hr. Each sample contained 0.5 mg of protein and Bromophenol Blue (0.1%) was used to monitor the moving front. The gels were stained with 0.1% amido black and destained with 4% acetic acid.

Quantitative analysis of α-arachin was performed according to Laurell (1966). All samples contained 10.0 μg of protein. The agar contained 2% antiserum against crude arachin. Electrophoresis proceeded for 12 hr at 200 V (20 V/cm). The dried plate was stained with 0.1% amido black and destained with 4.0% acetic acid.

Qualitative analysis of α-arachin and α_2-conarachin on a

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disc gel by immunodiffusion was done by allowing proteins to diffuse out of an acrylamide gel after electrophoresis into agar. Troughs perpendicular to the direction of diffusion were then filled with specific antiserum.

Immune serum was prepared by injections in three rabbits of a total protein extract containing 46 mg of protein/ml as follows.

Day	Volume, ml	Location
1	0.25	Hind foot pads
7	0.50	Back muscle
14	0.50	Back muscle
21	0.50	Back muscle
28 (bleeding—50 ml whole blood per rabbit)		

The immunizing antigens were emulsified in complete Freund's adjuvant before injection.

RESULTS

Identification of α-Arachin and α_2-Conarachin on Acrylamide Gels by Immunodiffusion. The original work on the characterization of the major peanut proteins by immunochemical techniques was reported by Daussant *et al.* (1969b) using specific antisera. Figure 1 shows the positions of α-arachin and α_2-conarachin on an acrylamide gel. The two bands corresponding to α-arachin have slightly different mobilities but show a continuous precipitin arc. These two zones were separated by starch gel electrophoresis and were shown to be antigenically identical by double diffusion (Neucere, 1971). α_2-Conarachin remains at the interface of the stacking gel and the running gel. Since the ratio of α_2- to α_1-conarachin is approximately 8 to 1 (Neucere and Ory, 1970), the quantity of α_1-conarachin is too low to give a visible precipitin line with the antiserum used here.

Immunoelectrophoresis. A preliminary study on the physicochemical changes of the peanut proteins induced by heat has been reported (Neucere *et al.*, 1969). The immunoelectrophoretic patterns of the proteins extracted from the heated samples in the present study are shown in Figure 2. The precipitin lines corresponding to α-arachin (α-arachin is the major protein of the arachin fraction; see Daussant *et al.*, 1969b) and one other antigen were persistent throughout the series of temperatures employed. Many of the antigenic sites were inactivated at a relatively low temperature (110°C) at high moisture content. Conversely, dry heat induced sequential antigenic degradation with increased temperature; α_2-conarachin remained antigenic up to 130°C (compare 2–6 with 7–11 of Figure 2). Note also that the precipitin lines for α-arachin in the imbibed seed (2–6, Figure 2) extended closer to the anode than the corresponding lines from the dry heated samples. This slight change in mobility implies a possible change in shape and/or conformation of this protein molecule, thus exposing interior negative functional groups.

Disc Electrophoresis. Electrophoreograms of the proteins from heated seeds are shown in Figure 3. In each case, 0.5 mg of protein was layered on top of the stacking gel. Approximately 12 components were observed in the control at this concentration. Of the major zones, B corresponds to polymeric forms of α-arachin, the major peanut globulin, and zone A corresponds to α_2-conarachin. The migrational effect of wet heat (2–6, Figure 3) was less obvious than on the dry-

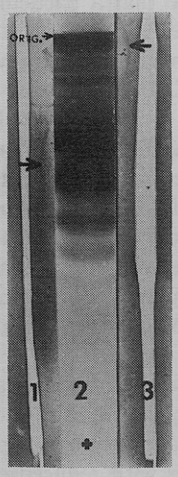

Figure 1. Identification of α-arachin and α_2-conarachin on acrylamide gels by immunodiffusion. Nomenclature: 1, trough containing immune serum directed against α-arachin; 2, disc gel of a total protein extract (control); 3, trough containing immune serum against α-conarachin

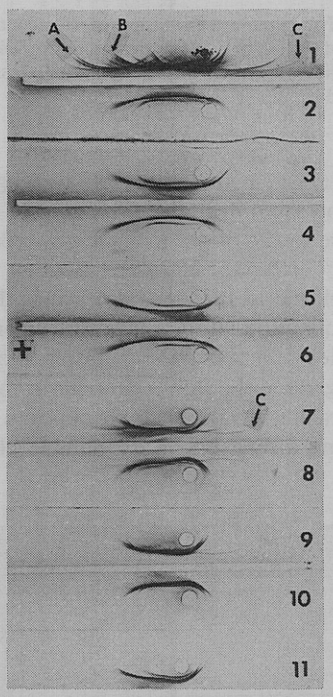

Figure 2. Immunoelectrophoreograms of the peanut proteins dissolved in phosphate buffer, pH 7.9, ionic strength 0.2. Nomenclature: 1, total proteins from unheated seed (control); A, α_1-conarachin, B, α-arachin, C, α_2-conarachin; 2–6, proteins extracted from meals of seeds imbibed 16 hr and heated 110, 120, 130, 145, and 155°C, respectively; 7–11, proteins extracted from meals of seeds dry-heated 110, 120, 130, 145, and 155°C, respectively

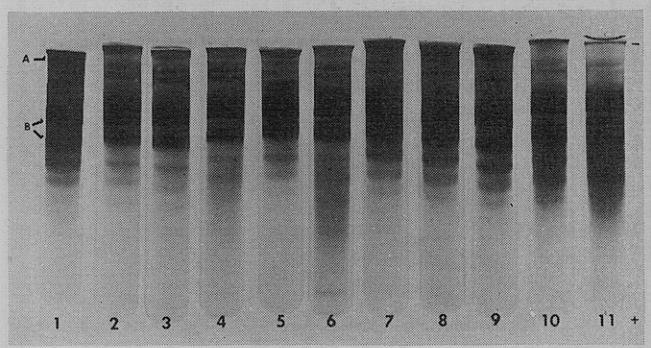

Figure 3. Disc electrophoresis of the protein samples described in Figure 2. Nomenclature: A, α_2-conarachin; B, polymeric forms of α-arachin. Migration is from top (cathode) to bottom (anode)

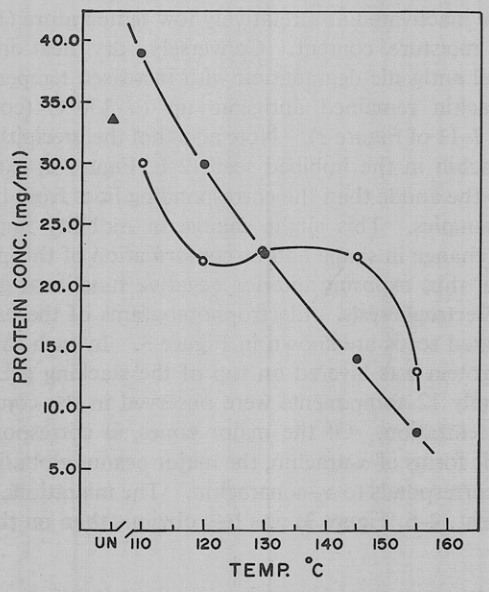

Figure 4. Relative protein solubilities of the control and heated samples described in Figure 2. Extraction procedure is given in experimental methods. Open circles, wet-heated samples; closed circles, dry-heated samples; triangle, control

heated samples (7–11, Figure 3). Note the slight increase in mobility with increased heat and the extent of depolymerization (zones close to the origin including α_2-conarachin) after dry heat at 130°C (10–11, Figure 3). Because the percent of α-arachin solubilized at higher temperatures decreased, perhaps the larger quantities of the faster moving proteins in each of these samples affect mobility; faster moving proteins would help drag along the slower proteins at the start and an increase in α-arachin would have an opposite effect. Since molecular sieving also exists in acrylamide electrophoresis,

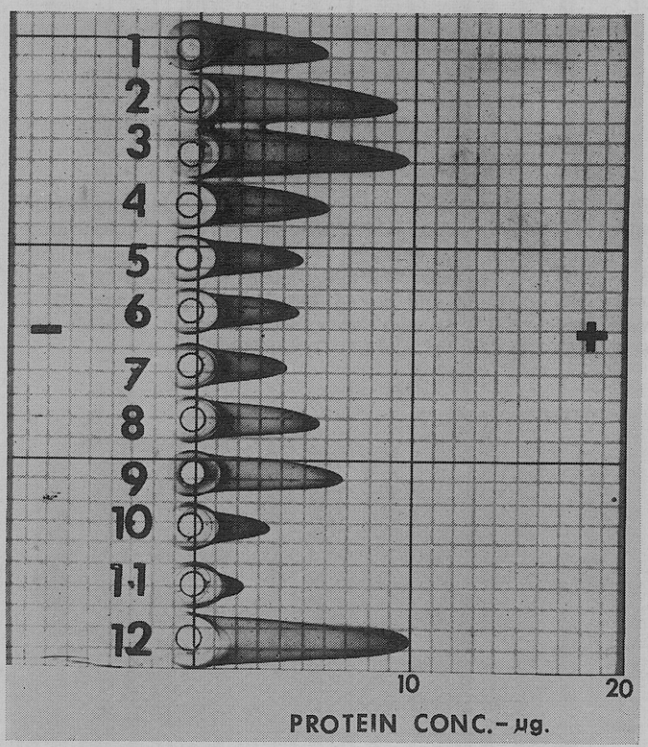

Figure 5. Semiquantitative analysis of α-arachin by electrophoresis in agar containing antibody. Sample numbers are identical to those in Figure 2 and sample 12 corresponds to arachin

maybe the release of small subunits at the higher temperatures also accounted in part for the observed changes in mobility.

Protein Solubility. Figure 4 shows the solubility curves of proteins extracted from both wet- and dry-heated seeds. Solubility decreased almost linearly with temperature for the dry-heated seed (closed circles). For the wet-heated samples, the relationship was quite different; a plot of solubility *vs.* temperature showed a sigmoid-like curve with a minimum at 120°C decreasing sharply after 145°C. Compared to the 110°C wet- and dry-heated samples, the control had an intermediate degree of solubility. Qualitatively, disc electrophoresis showed that wet-heated proteins were comparably soluble to native proteins at all levels of heat. Hence, failure to visualize specific precipitin reactions by immunoelectrophoresis only implied that the unique structure of certain antigenic sites was destroyed; the extent of solubility was not determined.

Semiquantitative Analysis of α-Arachin by Electrophoresis in Agar Gel Containing Antibody. The solubility curve in Figure 4 showed varying degrees in total protein solubility after heating; however, the amount of α-arachin solubilized at all levels of heat does not correlate directly. The analysis shown in Figure 5 is based on a technique described by Laurell (1966). In principle, the method involves the electrophoretic migration of an antigen which forms a solid complex with antibody imbedded in agar gel. The length of the conical peak formed is directly proportional to the concentration of antigen. The results showed maximum solubility of α-arachin after wet heat at 120°C (sample 3). For the seeds dry-heated, maximum solubility occurred at 130°C (sample 9). The relation between solubility and heat applied for the two sets of samples is parabolic, *i.e.*, intermediate heat increases the solubility of α-arachin.

DISCUSSION

Physical and chemical properties of proteins are best determined by considering all levels of structural organization. Clearly, the methods of analysis used in this study are not adequate to define precisely all levels of organization, especially from a mixture of proteins. Both electrophoresis and antigenic specificity, however, are useful tools in detecting changes relating to molecular size, shape, and charge, changes which can involve all levels of protein structure. It is imperative, also to realize that a particular treatment of proteins may, for example, drastically alter biological activity even though alternate measurements reveal only slight structural modifications. Considering disc electrophoretic and immunoelectrophoretic analyses, wet heat induced drastic changes in antigenic specificity of most of the proteins at low temperatures, which were not readily detectable by disc electrophoresis. Conversely, the accentuated migration differences of proteins from the dry-heated seeds were undetectable by immunoelectrophoresis since the proteins lost antigenic activity through conformational change. Hence, the most perplexing problem concerning protein denaturation is how to measure it.

The nature of the antigen–antibody reaction depends on the primary amino acid sequence and the secondary structure of the antigen. Studies have shown that proteins denatured by heat produce antibodies with reduced capacity to bind with native antigens (Avat, 1966). It appears, however, that immunological response cannot be generally described, since specificity is usually caused by more than one determinant group. Accordingly, the effects of heat on the determinant

groups of the native antigens from which the antibodies were elicited in this study varied for different proteins, some being completely inactivated. Much work has been done in an attempt to elucidate the mechanism of antibody formation. Rude *et al.* (1968), for example, have reported the correlation between net electrical charge on the antigen to that on the antibody it produces. Based on these observations, antibodies to acidic and basic antigens could be separated by ion exchange chromatography.

Protein hydration introduces a new environment that can elicit many changes in structural conformation. Perhaps the simplest reaction is the protonation of the protein molecule, which changes the overall electrostatic behavior. Klotz (1958) described the behavior of proteins in solution in terms of frozen water of hydration. Briefly, his hypothesis suggests mutual cooperation between water and protein molecules, imposing a fixed structure resembling an ice-like lattice. For such a model, the rigid lattice structure should decrease as the solution is heated. The effect of water and heat on proteins in the presence of other macromolecules, of course, is more complicated. Imbibition alone produces physiological changes after which biological activity is initiated. Marcus and Feeley (1965) have shown that ribosomes prepared from imbibed peanuts have the capacity to incorporate amino acids into protein, suggesting the formation or activation of M-RNA during imbibition. Hence, the entire biological structure of the seed is in dynamic flux shortly after water is introduced. Certainly, one would expect less heat stability in such a system, especially in enzymes which are functional in protein degradation and synthesis and in partially hydrolyzed storage proteins during the early stages of germination.

The stability of α-arachin might be attributed to its location within the cell. Electron microscopic studies of peanut parenchyma cells have been reported by Bagley *et al.* (1963) and Daussant *et al.* (1969b). α-Arachin is located in aleurone grains (protein bodies) which are surrounded by oil droplets in a matrix of cytoplasm. Perhaps both the oil and the membrane around the aleurone grains protect the orientation of the molecule somewhat by acting as a heat shield. Imbibition causes the aleurone grains to swell but degradation of these particles does not begin until the fourth day of germination (Bagley *et al.*, 1963); hence the particles remain intact at the time of heating.

Free radicals induced by radiation and peroxidizing lipids have been reported to alter proteins through the formation of both soluble and insoluble lipid–protein complexes and in disulfide production (Roubal and Tappel, 1966a,b). Perhaps similar reactions occurred during the heating of intact seeds and accounted in part for the observed changes in electrophoretic mobility, antigenicity, and in the overall change in protein solubility. Crosslinking of a variety of products within a cell could result in losses of biological function, as evidenced by loss of antigenicity.

The data make it clear that both moisture and temperature influence the reactivities of the peanut proteins. Since the three-dimensional structures for these proteins are unknown, however, it is difficult to directly associate their structures with the reactivities of specific functional groups. Any attempts to explain the observed changes must take into account not only conformational and environmental changes of proteins, but also the effects that can result from reactions involving other macromolecules and protein fragments.

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LITERATURE CITED

Avat, F., *Nature (London)* **212,** 848 (1966).
Bagley, B. W., Cherry, J. H., Rollins, M. L., Altschul, A. M., *Amer. J. Bot.* **50,** 523 (1963).
Daussant, J., Neucere, N. J., Conkerton, E. J., *Plant Physiol.* **44,** 480 (1969a).
Daussant, J., Neucere, N. J., Yatsu, L., *Plant Physiol.* **44,** 471 (1969b).
Davis, B. J., *Ann. N. Y. Acad. Sci.* **122,** 404 (1964).
Dechary, J. M., Talluto, K. F., Evans, W. J., Carney, W. B., Altschul, A. M., *Nature (London)* **190,** 1125 (1961).
Gorbacheva, L. B., *Biokhimiya* **22,** 70 (1957).
Grabar, P., Williams, C. A., *Biochim. Biophys. Acta* **10,** 193 (1953).
Haurowitz, F., in "The Chemistry and Function of Proteins," 2nd ed., Academic Press, New York, N.Y., 1965, p 163.
Johns, C. O., Jones, D. B., *J. Biol. Chem.* **28,** 77 (1916).
Johnson, P., Naismith, W. E. F., *Discuss. Faraday Soc.* **13,** 98 (1953).
Johnson, P., Shooter, E. M., *Biochim. Biophys. Acta* **5,** 361 (1950).
Johnson, P., Shooter, E. M., Rideal, E. J., *Biochim. Biophys. Acta* **5,** 376 (1950).
Jones, D. B., Horn, M. J., *J. Agr. Res.* **40,** 672 (1930).
Klotz, I. M., *Science* **128,** 813 (1958).
Laurell, C. B., *Anal. Biochem.* **15,** 45 (1966).
Lowry, O. H., Bosebrough, N. J., Farr, A. L., Randall, E. J., *J. Biol. Chem.* **193,** 265 (1951).
Marcus, A., Feeley, J., *J. Biol. Chem.* **240,** 1675 (1965).
Mikola, J., *Ann. Acad. Sci. Fenn. Ser. A2* **130,** 8 (1965).
Neucere, N. J., *Anal. Biochem.* **27,** 15 (1969).
Neucere, N. J., unpublished data, 1971.
Neucere, N. J., Ory, R. L., *Plant Physiol.* **45,** 616 (1970).
Neucere, N. J., Ory, R. L., Carney, W. B., J. AGR. FOOD CHEM. **17,** 25 (1969).
Roubal, W. T., Tappel, A. L., *Arch. Biochem. Biophys.* **113,** 5 (1966a).
Roubal, W. T., Tappel, A. L., *Arch. Biochem. Biophys.* **113,** 150 (1966b).
Rude, E., Mozes, E., Sela, M., *Biochemistry* **7,** 2971 (1968).
Tombs, M. P., Lowe, M., *Biochem. J.* **105,** 181 (1967).

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